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## Expression of the repulsive SLIT/ROBO pathway in the human endometrium and Fallopian tube

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### Abstract

We investigated whether the repulsive SLIT/ROBO pathway is expressed in the endometrium and is negatively regulated during implantation. We also examined whether deficient expression in the Fallopian tube (FT) may predispose to ectopic pregnancy (EP). Endometrium (n=21) and FT (n=17) were collected across the menstrual cycle from fertile women with regular cycles. FT (n=6) was obtained from women with EP and decidualised endometrium (n=6) was obtained from women undergoing termination. *SLIT/ROBO* expression was quantified by RT-PCR and protein localised by immunohistochemistry. The regulation of *SLIT/ROBO* expression *in vitro*, by sex steroids and hCG, was assessed in endometrial (hTERT-EEpC) epithelial cells, and the effects of *Chlamydia trachomatis* (CT) infection and smoking was studied in oviductal (OE-E6/E7) epithelial cells. Endometrial *SLIT3* was highest in the mid-secretory phase ( $P=0.0003$ ) and *SLIT1,2* and *ROBO1* showed a similar trend. *ROBO2* was highest in proliferative phase ( $P=0.027$ ) and *ROBO3,4* showed a similar trend. *SLIT2,3* and *ROBO1,4* were lower in decidua compared to mid-secretory endometrium ( $P<0.05$ ). *SLITs* and *ROBOs*, excepting *ROBO2*, were expressed in FT but there were no differences across the cycle or in EP. SLIT/ROBO proteins were localised to endometrial and FT epithelium. Treatment of hTERT-EEpC with a combination of estradiol and medroxyprogesterone acetate inhibited *ROBO1* expression ( $P<0.01$ ) but hCG had no effect. Acute treatment of OE-E6/E7 with smoking metabolite, cotinine, and CT had no effect. These findings imply a regulated role for the endometrial SLIT/ROBO interaction during normal development and pregnancy but that it may not be important in the aetiology of ectopic pregnancy.

### Keywords

Endometrium; Decidua; Ectopic pregnancy; *Chlamydia trachomatis*; Smoking

### Introduction

The Roundabout (ROBO) genes encode transmembrane receptors for their ligands, the SLIT proteins. There are three SLIT (1-3) proteins that bind to and activate each of the four

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ROBO (1-4) receptors (Legg *et al.*, 2008). The SLIT/ROBO pathway is widely expressed during development and one of its roles is to function as a repulsive cue in the regulation of cell migration (Dickinson and Duncan, 2010). It has an evolutionary conserved role in axon guidance during the development of the nervous system (Hinck, 2004). As well as being involved in the regulation of cell migration and angiogenesis (Jones *et al.*, 2008), the SLIT/ROBO interaction can promote cell death through apoptosis (Dallol *et al.*, 2005; Dickinson *et al.*, 2008). It therefore has particular roles in the development of major organs (Piper *et al.*, 2000; Hinck, 2004), including the ovary (Dickinson *et al.*, 2010). In addition, the various mutant mice suffer from multiple developmental abnormalities and the majority die *in utero* or shortly after birth (Xian *et al.*, 2001; Grieshammer *et al.*, 2004).

As the SLIT/ROBO interaction inhibits cell migration and promotes cell death, the SLITs and ROBOs also function as tumour suppressor genes (Dallol *et al.*, 2005; Chetodal *et al.*, 2005). There is increasing evidence for a loss of SLIT/ROBO expression, mainly through deletions and promoter region hypermethylation, in multiple cancers including those of the reproductive system (Dickinson *et al.*, 2004; Narayan *et al.*, 2006). We have recently shown however that SLITs and ROBOs also have a role in normal adult tissue physiology. We have reported expression of the SLIT/ROBO pathway in the human corpus luteum across the luteal phase of the menstrual cycle (Dickinson *et al.*, 2008). The expression of both the ligands (notably *SLIT2* and *SLIT3*) and receptors (notably *ROBO2*) peaked during luteolysis. In addition using *in vitro* human luteal cell models we showed that the luteotrophic molecules hCG (Myers *et al.*, 2007a) and cortisol (Myers *et al.*, 2007b) inhibited their expression. This suggests a regulated role in cyclical tissue remodelling in the ovary.

The uterus also undergoes tightly regulated cyclical remodelling under the control of systemic sex steroids and a number of other local factors (Jabbour *et al.*, 2006). During the menstrual cycle, the endometrium undergoes morphological and functional changes to prepare for implantation and pregnancy. However the paracrine interaction between the endometrial cells and the trophoblast to facilitate implantation is not yet fully understood. In 1.3-2% of all pregnancies aberrant implantation occurs in the Fallopian tube. Tubal ectopic pregnancy remains a major burden on health service resources (Wedderburn *et al.*, 2010) and continues to be associated with notable maternal morbidity and mortality (Farquhar, 2005). Why the blastocyst implants in the Fallopian tube is not clear as tubal ectopic pregnancy does not occur in other species (Corpa, 2006; Shaw *et al.*, 2010). There are however a number of known risk factors such as previous *Chlamydia trachomatis* infection and cigarette smoking (Faro, 1991; Bouyer *et al.*, 2003).

As the SLIT/ROBO interaction is a regulated pathway known to be involved in remodelling in the human ovary, we believe it may also have a functional role in the endometrium and Fallopian tube. As implantation involves paracrine signalling associated with cell survival and migration we hypothesised that the repulsive SLIT/ROBO interaction would be negatively regulated during implantation and that deficient expression in the Fallopian tube may predispose to ectopic pregnancy. This study therefore aimed to: 1) Examine the expression and localisation of the SLITs and ROBOs in human endometrium. 2) Determine if endometrial expression is inhibited at the time of implantation and during early pregnancy *in vivo* and study the effects of candidate regulatory molecules *in vitro*. 3) Examine the expression and localisation of the SLITs and ROBOs in the Fallopian tube. 4) Determine if their expression is inhibited in tubal ectopic pregnancy *in vivo* and study the acute effects of chlamydial infection and smoking *in vitro*.

## Materials and Methods

### Subjects and tissue collection

Ethical approval was obtained from Lothian Research Ethics Committee and written informed consent was obtained from all patients. Endometrial biopsies ( $n = 21$ ) were collected from fertile women with regular menstrual cycles (25–35 days) who had not undergone hormonal treatment in the previous three months as described previously (King *et al.*, 2009). These samples underwent histological dating (Noyes *et al.*, 1950) and were classified as menstrual ( $n=3$ ), proliferative ( $n=5$ ), early-secretory ( $n=4$ ), mid-secretory ( $n=5$ ) and late-secretory ( $n=4$ ). This corresponded to circulating serum estradiol and progesterone concentrations as described previously (King *et al.*, 2009). Biopsies from the ampullary region of the Fallopian tube were collected from a further 17 women undergoing hysterectomy for benign gynaecological conditions. All had regular menstrual cycles, no previous history of ectopic pregnancy and had not taken any exogenous hormone preparations in the three months prior to surgery. These biopsies were also dated with reference to endometrial histology and serum hormone concentrations. They were classified as menstrual ( $n=4$ ), follicular ( $n=5$ ) and mid-luteal ( $n=8$ ). In addition, Fallopian tube biopsies were obtained from six women undergoing surgical management of tubal ectopic pregnancy [mean gestation 58.09 days  $\pm$  SD 8.28; mean serum progesterone (P4) 58.53 nmol/l  $\pm$  SD 47.22]. None of these women presented acutely with haemodynamic shock and all required serial serum  $\beta$ hCG and ultrasound monitoring prior to diagnosis. Trophoblast-free decidual biopsies ( $n=6$ ) were obtained from women undergoing therapeutic surgical termination of pregnancy [mean gestation 69.75 days  $\pm$  SD 7.07, mean serum P4 61.71 nmol/L  $\pm$  SD 10.95] as described previously (Horne *et al.*, 2008).

A representative part of each biopsy was immersed in RNAlater storage solution (Ambion, Texas, USA) at 4°C overnight then stored frozen at  $-80^{\circ}\text{C}$ . A further equal part of the biopsy was fixed in 4% neutral buffered formalin overnight at 4°C, transferred to 70% ethanol and subsequently embedded into paraffin wax.

### RNA extraction and reverse transcription

RNA was extracted from frozen tissue biopsies using the QIAGEN RNeasy Mini Kit and QIAGEN Tissue Lyser (QIAGEN, Crawley, UK) according to manufacturer's instructions and quantified using a ND-1000 spectrophotometer (Nanodrop technologies, DE, USA). All samples were treated with DNaseI (QIAGEN) to remove genomic DNA contamination. Reverse transcription (RT) of RNA was performed in 30  $\mu$ l reaction volumes containing 1 $\times$ RT buffer, 25mM magnesium chloride, dNTPs, random hexamers, RNase inhibitor and Multiscribe reverse transcriptase (PE Applied Biosystems, Warrington, UK). RT-negative (containing template RNA but no reverse transcriptase enzyme) and RT water (containing reverse transcriptase but no template RNA) were included in every cDNA reaction as negative controls.

### Quantitative Real-Time PCR (Q-RT-PCR)

Taqman Q-RT-PCR was used to measure gene expression levels. Specific primers were designed using the Universal Probe Library Assay Design Center ([www.roche-applied-science.com](http://www.roche-applied-science.com)) and used in conjunction with Universal Probe Library (UPL) probes (Roche Applied Science). Details of primer sequences and probes used are given in Table 1. All reactions were performed in triplicate using an ABI 7900 sequence detection system. Using the  $2^{-\Delta\Delta\text{Ct}}$  method, mRNA expression results were normalised against ribosomal 18S internal control (PE Applied Biosystems, Oxford, UK) and expressed as relative expression compared to cellular or positive tissue controls.

## Immunohistochemistry

Pilot studies showed we could reliably immunolocalise SLIT2, ROBO1, ROBO2 and ROBO4 using polyclonal antibodies (SantaCruz Biotechnology, Santa Cruz, CA, USA; Abcam, Cambridge, UK) and peptide-block controls. These proteins (excepting ROBO2 in the Fallopian tube) were therefore immunolocalised by standard methods using biotinylated secondary antibodies and ABC-Elite avidin biotin peroxidase complex (Vector Laboratories, Peterborough, UK) (Dickinson *et al.*, 2010). Briefly, 3µm paraffin sections of endometrium and Fallopian tube were cut, dewaxed in xylene, rehydrated and subjected to antigen retrieval by microwaving in Antigen Unmasking Solution (Vector Laboratories) before blocking endogenous peroxidase with 3% hydrogen peroxidase (Sigma, Dorset, UK). An avidin-biotin block (Vector Laboratories) and protein block (Dako, Ely, UK) were performed prior to overnight incubation with primary antibodies. Negative controls were included in every run where the primary antibody was pre-incubated with a blocking peptide. Sections were then incubated with biotinylated secondary antibody (Vector Laboratories) and ABC-Elite (Vector Laboratories). Positive immunostaining was visualised using 3,3-diaminobenzidine (Vector Laboratories). The stained sections were then counterstained in haematoxylin, mounted in Pertex (Cellpath PLC, Hemel Hempstead, UK) and compared to negative control sections.

## Cell Culture

The immortalised endometrial epithelial cell line (hTERT-EEpC; Hombach-Klonisch *et al.*, 2005) was used as an *in vitro* model to investigate the effects of steroid hormones and hCG. Cells were maintained in Ham's F-12 media (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum and transferred to serum-free medium overnight prior to treatments. The cells were treated with concentrations of hCG (10 and 100 IU/ml; Organon Laboratories, Cambridge, UK) mimicking those at the maternal epithelia adjacent to an implanting embryo (Horne *et al.*, 2009a; Nakayama *et al.*, 2003). All experiments were conducted in triplicate and cells were collected after 6 and 24 hours. These time points were established by our previous studies (Horne *et al.*, 2009a). In addition the cells were treated with estradiol (E2) (10nM), medroxyprogesterone acetate (MPA) (1µM), vehicle control or a combination of E2 (10nM) and MPA (1µM) for 24 hours. This treatment regimen has previously been validated as an *in vitro* model of steroid action and utilised standard, and relevant, concentrations of steroids (Horne *et al.*, 2006). Each experiment was conducted three times and cells were collected after 24 hours.

The OE-E6/E7 oviductal epithelial cell line (Lee *et al.*, 2001) was used to assess the effects of smoking and chlamydial infection. Cells were maintained in Dulbecco's modified Eagle's medium F12 (Invitrogen Ltd, Paisley, UK) containing 10% fetal calf serum and plated in 48-well culture plates at a density of  $2 \times 10^5$  cells/well. Prior to treatment cells were transferred to serum-free medium overnight. Treatments were then applied in a 500µl volume and performed in triplicate. Cells were exposed to live *C. trachomatis* at multiplicities of infection (MOI) of 0.001, 0.01 and 0.1 for 24 or 48 hours. To control for the presence of modulatory factors in the oviductal cell lysate resulting from infection, cells were also exposed to cell lysate (MOI 0). Uninfected cells were included as additional controls.

To assess the effect of smoking we utilised an *in vitro* approach where cells were treated with cotinine, a metabolite of nicotine (Conklin *et al.*, 2002). Cells were plated in 12-well culture plates at a density of  $2 \times 10^5$  cells/well and transferred to serum-free medium overnight prior to treatment. Cells were treated with cotinine (Sigma) at a concentration of either 40ng/mL (representing the concentrations achieved in passive smoking) or 400ng/mL (representing the concentrations achieved in heavy regular smoking) (Haddow *et al.*, 1987) or carrier control. Experiments were carried out three times and cells were collected at 4, 8,

12 and 24 hours. In all culture experiments the cells were collected for subsequent RNA extraction and analysis of mRNA expression by Q-RT-PCR as described previously. In these experiments we focussed on assessing the effects on the expression of the primary ROBO in these cells, *ROBO1*, and the representative SLIT, *SLIT2*.

### Statistical analysis

Statistical analysis was conducted using a Prism software package (GraphPad Software Inc., La Jolla, CA, USA) with significance defined as  $P < 0.05$ . Analysis was performed using one-way analysis of variance and Bonferroni's post-hoc test where three or more variables were being compared. A t-test was used when two variables were being compared. Where there were significant differences in variances the data were logarithmically transformed to ensure normal distribution in all cases prior to statistical analysis.

## Results

### SLIT/ROBO genes are expressed in human endometrium

Messenger RNA for all known genes in the SLIT/ROBO family was expressed in human endometrium (Fig. 1). The pattern of expression for each of the *SLIT* genes was similar across the menstrual cycle with maximal expression in the mid-secretory phase (Fig. 1a,c,e). This reached statistical significance for *SLIT3* ( $P = 0.0003$ ). The pattern of expression across the menstrual cycle for *ROBO2*, *ROBO3* and *ROBO4* was similar, although it was different to that of the *SLITs* (Fig. 1i,k,m). The peak *ROBO2*, *ROBO3* and *ROBO4* expression was seen in the proliferative phase and this reached statistical significance for *ROBO2* ( $P = 0.027$ ). Interestingly the pattern of *ROBO1* expression was more similar to the *SLITs* than the other *ROBOs* (Fig. 1g). Overall this suggests that endometrial expression of the SLIT/ROBO system is hormonally regulated and may have a role in the endometrial function.

### SLIT/ROBO gene expression changes after decidualisation

The expression of *SLIT2* ( $P < 0.05$ ), *SLIT3* ( $P < 0.005$ ), *ROBO1* ( $P < 0.05$ ) and *ROBO4* ( $P < 0.0005$ ) was reduced in the decidualised endometrium of pregnancy when compared to the mid-secretory phase (Fig. 1). Unlike other members of the SLIT/ROBO family, *SLIT1* ( $P < 0.05$ ) expression, was very low in the non-pregnant endometrium and increased in the decidualised endometrium of early pregnancy (Fig. 1b). This pattern is consistent with an inhibition of the endometrial SLIT/ROBO repulsive system during the establishment of pregnancy.

### SLIT and ROBO proteins are localised to the endometrial epithelium

Immunohistochemistry was used to examine the endometrial localisation of candidate SLIT and ROBO proteins (Fig. 2). SLIT2 and ROBO1 were predominantly expressed in the surface and glandular epithelium in the human endometrium. ROBO1 (Fig. 2b) and to a lesser extent SLIT2 (Fig. 2a) could also be detected in individual stromal cells near the surface epithelium. ROBO2 and ROBO4, the ROBO proteins that demonstrated a slightly different pattern of mRNA expression across the cycle, were also primarily expressed by epithelial cells (Fig. 2e,f). ROBO4 could also be localised to stromal cells that appeared to be endothelial in nature (Fig. 2f). The SLIT/ROBO system is therefore expressed in endometrial epithelial cells at the stage of the cycle when implantation occurs.

### SLIT/ROBO genes are expressed in human Fallopian tube

Expression of the *SLIT* and *ROBO* genes, with the exception of *ROBO2*, could also be detected in the Fallopian tube across the menstrual cycle (Fig. 3). Although again there was



a similar pattern of *SLIT2* (Fig. 3c) and *ROBO1* (Fig. 3b) expression, there were no statistical differences in expression of any of the *SLIT* or *ROBO* genes across the menstrual cycle (Fig. 3). In addition there was no difference in the levels of expression of the *SLIT/ROBO* genes in the Fallopian tubes of patients with tubal ectopic pregnancy (Fig. 3). This suggests that there may be no reduction in the expression of the *SLIT/ROBO* system in tubal implantation.

### **SLIT/ROBO proteins are expressed by the tubal epithelium**

Although there was some faint immunostaining in some smooth muscle and stromal cells, the primary site of *SLIT2* and *ROBO1,4* expression in the Fallopian tube, in both the follicular and luteal phases of the cycle, was the epithelium (Fig. 4). Again the *SLIT/ROBO* system seems to be expressed at the epithelial site of ectopic attachment of the conceptus in the oviduct. However the localisation of *SLIT* and *ROBO* in the Fallopian tubes from women with a tubal ectopic pregnancy was similar to that of the non-pregnant tubes (Fig. 4e,f).

### **Regulation of SLIT/ROBO expression in the endometrial epithelial cells**

In order to investigate factors involved in the regulation of the *SLIT/ROBO* pathway in the decidua an immortalised endometrial epithelial cell line was studied *in vitro*. Neither E2 alone, MPA alone nor a combination of E2 and MPA had any effect on the expression of *SLIT2* over 24 hours (Fig. 5a). *ROBO1* was not regulated by either E2 or MPA but a combination of E2 and MPA inhibited its expression over 24 hours ( $P<0.01$ ) (Fig. 5b). Treatment with either 10 or 100IU/mL hCG showed no effect on either *SLIT2* (Fig. 5c) or *ROBO1* (Fig. 5d) expression after 6 and 24 hours of culture.

### **Regulation of SLIT/ROBO expression in oviductal epithelial cells by known risk factors for ectopic pregnancy**

In order to determine if known risk factors for tubal ectopic pregnancy could regulate the expression of the *SLIT/ROBO* pathway in tubal epithelial cells an *in vitro* model was used to investigate the acute effects of smoking and chlamydial infection. The effect of smoking on the Fallopian tube was modelled using an oviductal epithelial cell line exposed to physiological concentrations of cotinine. Treatment with a low dose (40ng/mL; representative of passive smoking) or a high dose (400ng/mL; representative of regular smoking) had no effect on the expression of either *SLIT2* or *ROBO1* at any of the time points over the 24 h assessment (Fig. 5e,f).

The acute effect of *C. trachomatis* infection on the *SLIT/ROBO* pathway in the Fallopian tube was then investigated using this oviductal epithelial cell line (Fig. 5g,h). There was no effect on the expression of *SLIT2* following infection with *C. trachomatis* after 24 or 48 hours (Fig. 5g). However there seemed to be a bimodal effect on the expression of *ROBO1*. After 24 hours there was a trend for an increase in expression but after 48 hours the expression of *ROBO1* tended to be lower than the control cells. At the highest Chlamydia inoculation titres there was a significant reduction in *ROBO1* expression between 24 and 48 hours exposure ( $P<0.05$ ) (Fig. 5h).

## **Discussion**

To our knowledge this is the first comprehensive report of the patterns of expression of the *SLIT/ROBO* system in the human endometrium and Fallopian tube at different phases of the menstrual cycle. We also report on *SLIT/ROBO* expression at normal endometrial and pathological tubal implantation sites. In addition, we have used an *in vitro* model system to

investigate the impact of acute exposure to factors thought to influence implantation on SLIT/ROBO expression in these tissues.

We demonstrate that all of the known *SLITs* and *ROBOs* are expressed in the endometrium across the menstrual cycle. Only the expression of *SLIT3*, significantly changed across the menstrual cycle but each of the *SLITs* followed the same pattern with maximal expression in the mid-secretory phase. As each of the *SLITs* seems to have the same action at each of the *ROBO* receptors this suggests a peak of repulsive ligand expression at the time of expected implantation. This implies that their expression is hormonally regulated and that the *SLITs* may have a specific role in endometrial function. However, unlike the corpus luteum (Dickinson *et al.*, 2008), in the endometrium cell death does not follow the pattern of *SLIT* expression. In the endometrium, cell death increases in the late-secretory phase and is highest during the menstrual phase (Otsuki, 2001; Jabbour *et al.*, 2006). Endometrial tissue remodelling and angiogenesis however occurs after menstruation, is primarily driven by estradiol and vascular endothelial growth factor and is largely complete by the mid-secretory phase (Fraser *et al.*, 2008). It is therefore possible that the peak in *SLIT* expression at the mid-secretory phase may reflect or influence this process (Legg *et al.*, 2008). However, the nature of the role for *SLIT* in the endometrium remains unclear.

In the corpus luteum, the expression of *ROBOs* mimics the pattern of expression of the *SLITs* (Dickinson *et al.*, 2008). While this was true in the endometrium for *ROBO1* and *SLIT1*, it was not for the other *ROBOs*. In the fetal ovary, the *SLITs* and *ROBOs* also show a discrepancy in their pattern of expression (Dickinson *et al.*, 2010). The nature of this differential expression is unclear but it does suggest that regulation of *ROBOs* is different to that of the *SLITs* in both tissues. However there are receptors to the *SLIT* ligands in the endometrium across the menstrual cycle.

Another interesting feature of the *ROBOs* was the endometrial stromal cell immunostaining. It has been reported that vascular endothelial cells express *ROBO4* (Park *et al.*, 2003; Legg *et al.*, 2008), and our findings suggest that this is likely to be the case in the human endometrium but we also demonstrate specific epithelial staining. While *SLIT2* could be detected in some individual cells in the stroma, this was particularly obvious for *ROBO1*. The nature of those specific cells is not clear but they may represent immune cells. Certainly the *SLIT/ROBO* pathway has previously been described in lymphocytes (Prasad *et al.*, 2007). However, these undefined immunostained cells were particularly marked in the proliferative phase and it is the mid-secretory stage that the immune population peaks with the accumulation of uterine CD56+ve natural killer cells (King, 2000; Jabbour *et al.*, 2006). While it is attractive to speculate that immune cells are involved in the *SLIT/ROBO* interaction in the endometrium, further work needs to be done to confirm this.

As steroids are the primary regulatory molecules controlling the human endometrium (Jabbour *et al.*, 2006) we hypothesised that they would regulate *SLIT/ROBO* expression. Certainly there are steroid response elements on the promoter regions of these genes (Dickinson RE, unpublished observations) and glucocorticoids inhibit both *SLIT* and *ROBO* expression in ovarian cells (Dickinson *et al.*, 2008). In addition there are steroid receptors on uterine immune cell populations (Henderson *et al.*, 2003). As progesterone receptors are lost from endometrial glandular epithelium in the secretory phase (Koh *et al.*, 1995) it is possible that progesterone is suppressive and that the absence of progesterone receptors is responsible for the peak in expression at this time. However, MPA alone did not suppress *SLIT2* or *ROBO1* and E2 in combination with MPA did not suppress *SLIT2 in vitro*. Nonetheless, we did discover that *ROBO1* expression was inhibited by the combination of E2 and MPA. The biological significance of this, and whether the addition of E2 promotes progesterone receptor expression (Jabbour *et al.*, 2006), is uncertain and requires further



study. Recently it has been suggested that SLIT/ROBO1 is increased in ovarian endometriomas (Shen *et al.*, 2009). Sex steroid receptors however are maintained or increased in ectopic endometrium (Fujishita *et al.*, 1997; Jones *et al.*, 1998). Although it remains possible that steroids are involved in the physiological regulation of endometrial SLIT/ROBO we have not been able to fully confirm this *in vitro*.

We speculated that the SLIT/ROBO system would have a role in the regulation of implantation. If the system functions as a repulsive cue, that can be regulated, we hypothesised that endometrial expression of *SLITs* or *ROBOs* would be down-regulated during implantation. Although there was no down regulation during the mid-secretory implantation window we found some evidence for this in early gestation. The decidualised endometrium of pregnancy demonstrated a significant reduction in the expression of *SLIT2*, *SLIT3*, *ROBO1* and *ROBO4*. The factors involved in regulating this expression are not clear but *in vitro* the decidual is exposed to a combination of estrogen and progesterone as well as increased local cortisol, as a consequence of *HSD11B1* expression (Michael *et al.*, 2003; McDonald *et al.*, 2006). Increased local cortisol is associated with reduced *SLIT/ROBO* expression in the ovary (Dickinson *et al.*, 2008). It is not yet known whether trophoblast cells express *SLIT* or *ROBO* or if trophoblast products can regulate endometrial *SLIT* or *ROBO* expression. We found no evidence for acute regulation by hCG using a treatment regimen that has been shown to influence endometrial gene expression (Horne *et al.*, 2009a). It is likely that there is some regulation of the SLIT/ROBO interaction during intrauterine implantation but the importance and cause of this regulation requires further study.

We have also shown that the *SLITs* and *ROBOs*, with the exception of *ROBO2* are expressed in the Fallopian tube and SLIT/ROBO protein is localised to the tubal epithelium. However, unlike the endometrium there was no change in mRNA expression across the cycle. Differential regulation of specific proteins in the Fallopian tube compared to endometrium has also been shown for sex steroid receptors. In contrast to the endometrium, epithelial steroid receptors are consistently expressed in the Fallopian tube across the menstrual cycle (Amso *et al.*, 1994; Horne *et al.*, 2009b).

It is not clear if the expression of SLIT and ROBO in the tubal epithelium provides a repulsive cue to tubal implantation. We did not detect any changes in SLIT/ROBO expression in the Fallopian tubes of ectopic pregnancy compared to the non-pregnant Fallopian tube. This might imply that there is no local down regulation of *SLITs* or *ROBOs* in the Fallopian tube by the implanted embryo in contrast to that seen in the endometrium. However, it is not possible, for ethical reasons, to study the expression of the *SLITs* and *ROBOs* in the Fallopian tube of a woman with an intrauterine pregnancy. Thus, it is feasible that the reduced sex steroid receptor expression that has been recently observed in the Fallopian tube of women with ectopic pregnancy (Horne *et al.*, 2009b) may also be seen in the Fallopian tube in an intrauterine pregnancy.

Thus, the function of the SLIT/ROBO system in the Fallopian tube is unclear. We hypothesised that, if their function is to inhibit implantation/invasion of ectopic trophoblast or to affect embryo transport, their expression would be altered by components known to act as risk factors for ectopic pregnancy such as smoking or chlamydial infection (Farquhar, 2005). We assessed the effect of smoking by exposing oviductal epithelial cells to cotinine, a bioactive metabolite of nicotine. Cotinine has a much longer half-life than nicotine and has a similar effect to nicotine *in vitro* (Conklin *et al.*, 2002). In addition, salivary or serum concentrations of cotinine have been widely used to establish and quantify an individual's exposure of smoking (Haddow *et al.*, 1987). We were therefore able to assess the effects of biologically relevant concentrations of cotinine. We focussed our Fallopian tube studies on the representative *SLIT2* and *ROBO1* molecules and did not see any acute direct effects of

cotinine on their expression. We have not assessed the effect of other constituents of cigarette smoke (Rodgman *et al.*, 2000), prolonged exposure, hypoxia, or the impact of other cells such as immune cells in this study and it remains possible that there are effects *in vivo*. However we have not been able to support the hypothesis that the effect of smoking on the risk of ectopic pregnancy is mediated through acute regulation of *SLIT* or *ROBO* expression.

We also tested whether an acute exposure to *C. trachomatis* could regulate *SLIT2* or *ROBO1* expression. We did not detect any changes to *SLIT2* expression after infection with increasing concentrations of *Chlamydia*. However, there seemed to be an increase in *ROBO1* expression at the highest infective dose after 24 hours although this was reduced after 48 hours. To determine whether this is a relevant or important observation requires further investigation as well as more prolonged study and a comparison of Fallopian tubes with and without chlamydial infection *in vivo*. In addition we acknowledge that immune cells, missing from this model, may mediate some of the endothelial effects of Chlamydia infection.

To summarise, the demonstration in the human endometrium of temporal expression of *SLITs* and *ROBOs* across the menstrual cycle and of local down regulation during intrauterine implantation implies an important role for the *SLIT/ROBO* interaction during normal endometrial development. This is further evidence that these developmentally important tumour suppressor genes have physiological roles, with regulated expression, in the adult female reproductive tract. However, we found consistent expression of *SLITs* and *ROBOs* in the Fallopian tube across the menstrual cycle and in Fallopian tube from women with ectopic pregnancy. This and the lack of clear regulation *in vitro* by cotinine or *Chlamydia*, do not support our hypothesis that the repulsive *SLIT/ROBO* system in the Fallopian tube is dysregulated and implicated in pathological tubal implantation.

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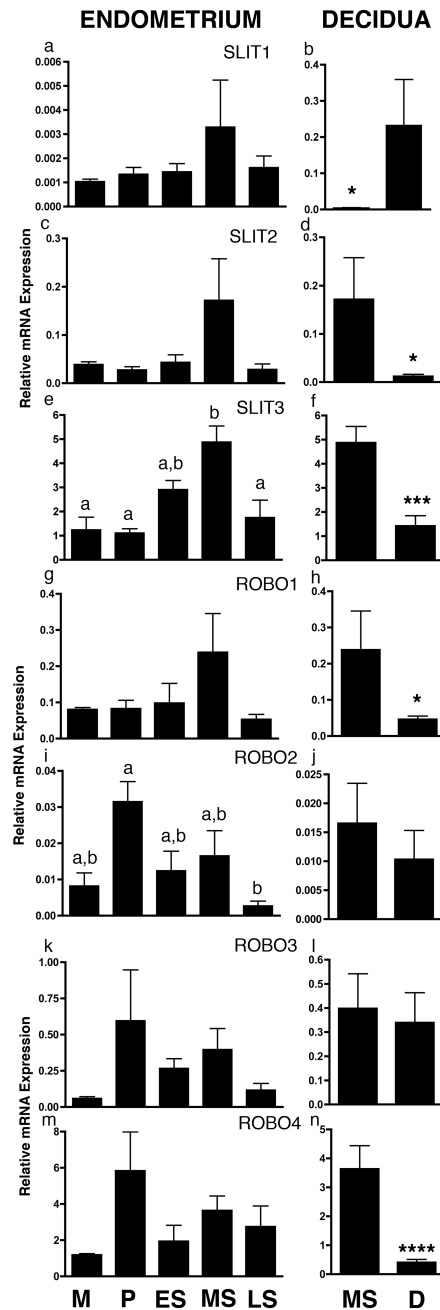
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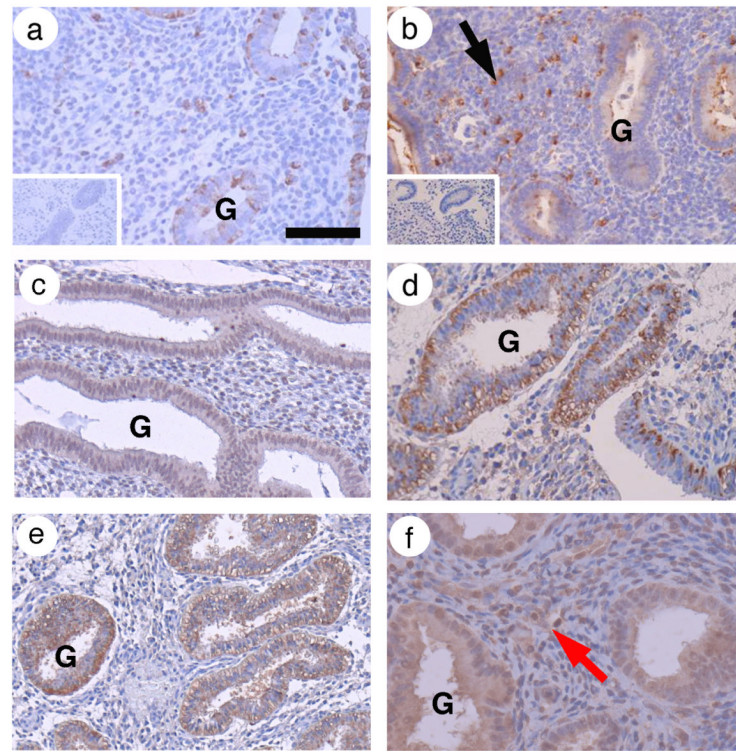
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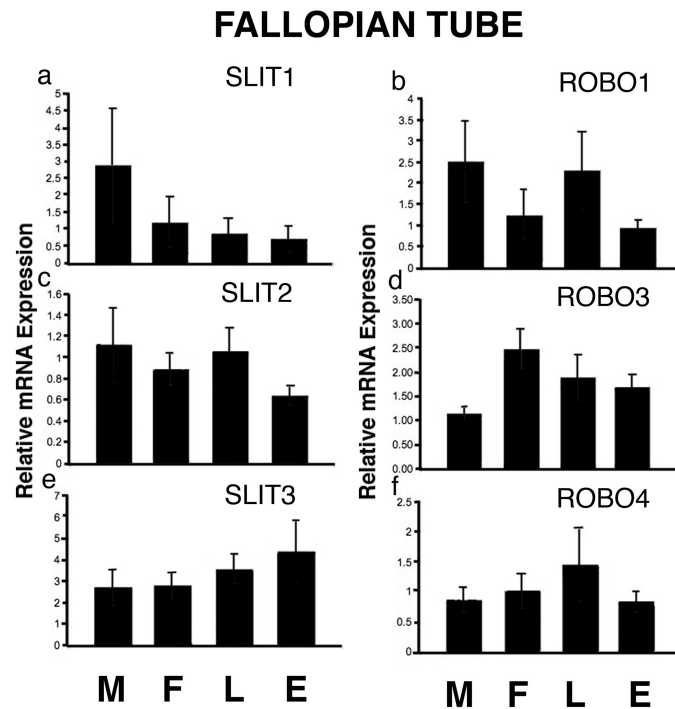
**Figure 1.**

Expression of *SLIT1-3* and *ROBO1-4* mRNA in endometrium across the menstrual cycle (a,c,e,g,i,k,m) and in the decidua of early pregnancy (b,d,f,h,j,l,n). The stages of the cycle are menstrual (M), proliferative (P), early-secretory (ES), mid-secretory (MS), late-secretory (LS) and decidua of early pregnancy (D). Different letters represent significant differences ( $P < 0.05$ ), \* represents  $P < 0.05$ , \*\*\* represents  $P < 0.005$  and \*\*\*\* represents ( $P < 0.0005$ ).



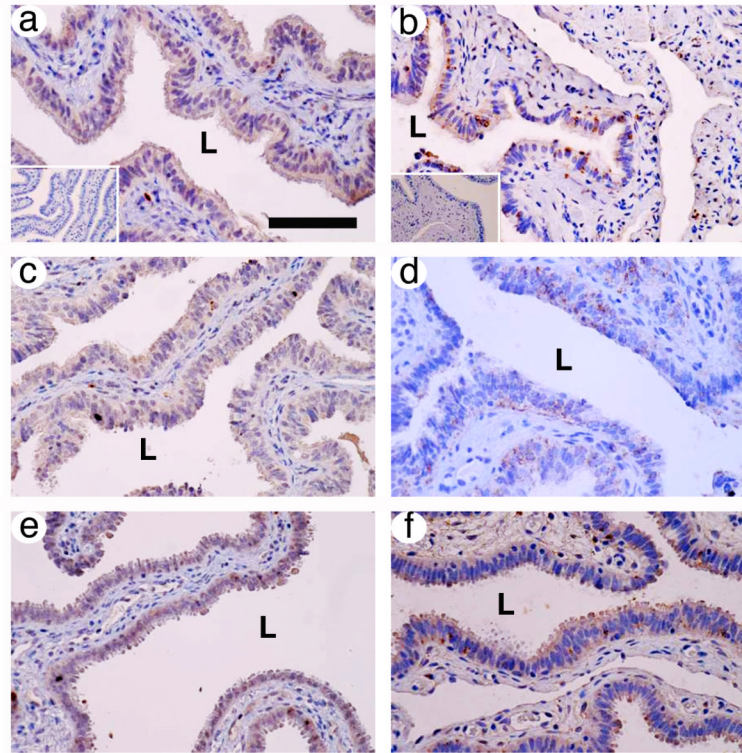
**Figure 2.**

Representative immunolocalisation of SLIT and ROBO proteins (brown) in human proliferative and mid-secretory endometrium. a) SLIT2 in proliferative phase endometrium. b) ROBO1 in proliferative phase endometrium where one of the individually stained stromal cells (black arrow) has been highlighted. c) SLIT2 in secretory phase endometrium. d) ROBO1 in secretory phase endometrium. e) ROBO2 in secretory phase endometrium. f) ROBO4 in proliferative phase endometrium where the likely endothelial staining in the stroma has been highlighted (red arrow). Negative controls are seen in the insets and the glandular epithelium is labelled (G). Scale bar represents 100  $\mu\text{m}$  (b-e), 75  $\mu\text{m}$  (a) 50  $\mu\text{m}$  (f).



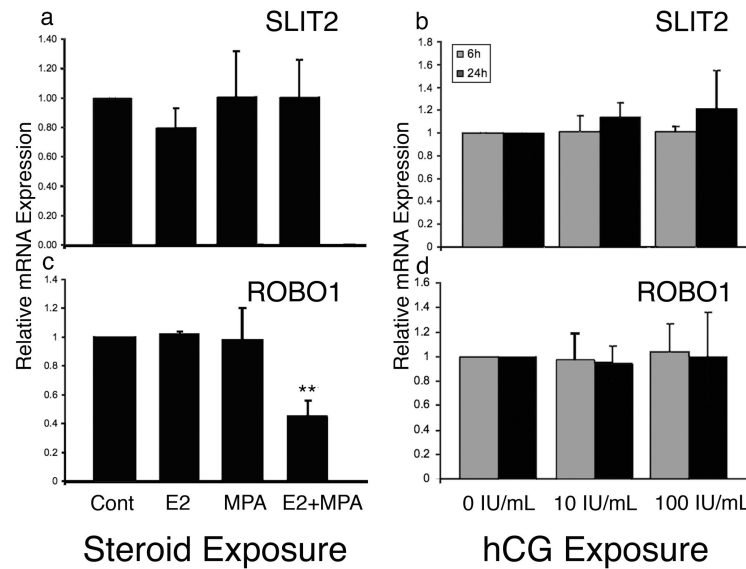
**Figure 3.**

Expression of *SLIT1-3* and *ROBO1,3&4* mRNA in Fallopian tubes across the menstrual cycle and in Fallopian tubes from women with ectopic pregnancy. The stages of the cycle are menstrual (M), follicular (F), luteal (L) and ectopic (E). There were no significant differences in expression ( $P>0.05$ ).



**Figure 4.**

Representative immunolocalisation of SLIT2 and ROBO1 proteins (brown) in human Fallopian tube. a) SLIT2 in follicular phase Fallopian tube. b) ROBO1 in follicular phase Fallopian tube. c) SLIT2 in luteal phase Fallopian tube. d) ROBO1 in luteal phase Fallopian tube. e) SLIT2 in the tube collected from an ectopic pregnancy. f) ROBO1 in the tube collected from an ectopic pregnancy. Negative controls are seen in the insets and the tubal lumen is labelled (L). Scale bar represents 100  $\mu\text{m}$ .



**Figure 5.**

Manipulation of *SLIT2* and *ROBO1* expression *in vivo*. a,b) The effect of estrogen (E2: 10 nM estradiol) and medroxyprogesterone acetate (MPA: 10 nM) in endometrial epithelial cells (hTERT EEpCs). a) *SLIT2* expression does not change after steroid exposure for 24h. b) *ROBO1* expression is reduced (\*\* $P < 0.01$ ) by a combination of E2 and MPA for 24h. c,d) The effect of hCG (10 IU/ml and 100 IU/ml) in hTERT EEpCs. c) *SLIT2* and d) *ROBO1* are not altered by hCG after 6h (grey bars) or 24h (black bars). d) *ROBO1* is also not altered by hCG after 6h (grey bars) or after 24h (black bars). e,f) The effect of cotinine in oviductal epithelial cells. There were no significant differences ( $P > 0.05$ ) of treatment with cotinine at 40 ng/ml (grey bars) and 400 ng/ml (white bars) on the expression of *SLIT2* (e) and *ROBO1* (f) relative to control (black bars) at four different time points up to 24 hours. g,h) The effect of *C. trachomatis* exposure in oviductal epithelial cells at different multiplicities of infection (MOI) for 24 (grey bars) or 48 (black bars) hours on the relative expression of *SLIT2* (g) and *ROBO1* (h). A significant difference ( $P < 0.05$ ) in *ROBO1* expression between 24 and 48 hours exposure to the highest infective concentration was noted.



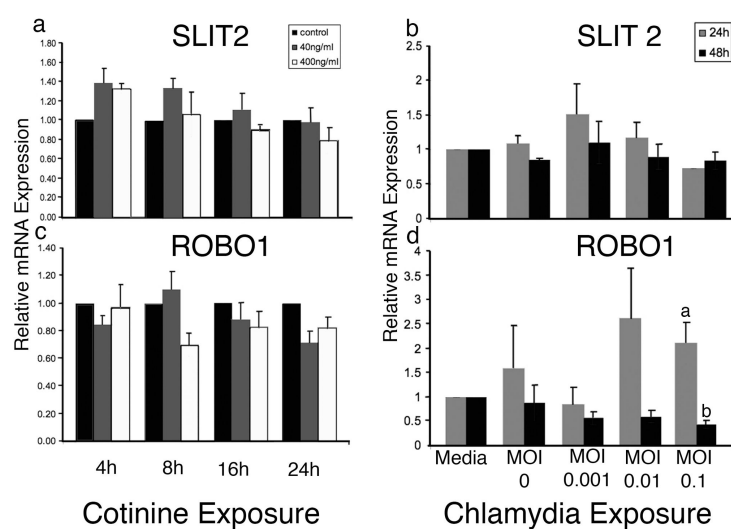


Figure 6.

**Table 1**

Primer and Probe combinations used for Taqman Q-RT-PCR.

	<b>Forward Primer</b>	<b>Reverse Primer</b>	<b>Accession Number</b>	<b>Probe Number</b>
<b><i>ROBO1</i></b>	aaagtagcacgacggcaaat	ggcactgagacgcatgaaa	NM_133631.1	89
<b><i>ROBO2</i></b>	tttatatgttcgggtgatgga	atgacatcggaaggatgctc	NM_002942.3	79
<b><i>ROBO3</i></b>	cggagtcggagtcagagc	acaagggtcatcttggtcct	NM_022370.2	21
<b><i>ROBO4</i></b>	catcatccgtggctaccag	cagttggctgggtggcagt	NM_019055.4	61
<b><i>SLIT1</i></b>	atgagccagctgaccactct	gagcggagtcctcctggaag	NM_003061.2	73
<b><i>SLIT2</i></b>	catggaggaaactgccactta	tccatcagcacaatacacca	NM_004787.1	12
<b><i>SLIT3</i></b>	cgagcactgccacaaga	ggcacatgatgcataaccttt	NM_003062.1	70